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Acute and Chronic Toxicity of Sodium Nitrate and Sodium Sulfate to Several Freshwater Organisms in Water-Only Exposures

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Abstract: Elevated nitrate (NO_3) and sulfate (SO_4) in surface water are of global concern, and studies are needed to generate toxicity data to develop environmental guideline values for NO_3 and SO_4. The present study was designed to fill existing gaps in toxicity databases by determining the acute and/or chronic toxicity of NO_3 (tested as $NaNO_3$) to a unionid mussel (*Lampsilis siliquoidea*), a midge (*Chironomus dilutus*), a fish (rainbow trout, *Oncorhynchus mykiss*), and 2 amphibians (*Hyla versicolor* and *Lithobates sylvaticus*), and to determine the acute and/or chronic toxicity of SO_4 (tested as Na_2SO_4) to 2 unionid mussels (*L. siliquoidea* and *Villosa iris*), an amphipod (*Hyalella azteca*), and 2 fish species (fathead minnow, *Pimephales promelas* and *O. mykiss*). Among the different test species, acute NO_3 median effect concentrations (EC50s) ranged from 189 to >883 mg NO_3-N/L, and chronic NO_3 20% effect concentrations (EC20s) based on the most sensitive endpoint ranged from 9.6 to 47 mg NO_3-N/L. The midge was the most sensitive species, and the trout was the least sensitive species in both acute and chronic NO_3 exposures. Acute SO_4 EC50s for the 2 mussel species (2071 and 2064 mg SO_4/L) were similar to the EC50 for the amphipod (2689 mg SO_4/L), whereas chronic EC20s for the 2 mussels (438 and 384 mg SO_4/L) were >2-fold lower than the EC20 of the amphipod (1111 mg SO_4/L), indicating the high sensitivity of mussels in chronic SO_4 exposures. However, the fathead minnow, with an EC20 of 374 mg SO_4/L, was the most sensitive species in chronic SO_4 exposures whereas the rainbow trout was the least sensitive species (EC20 > 3240 mg SO_4/L). The high sensitivity of fathead minnow was consistent with the finding in a previous chronic Na_2SO_4 study. However, the EC20 values from the present study conducted in test water containing a higher potassium concentration (3 mg K/L) were >2-fold greater than those in the previous study at a lower potassium concentration (1 mg K/L), which confirmed the influence of potassium on chronic Na_2SO_4 toxicity to the minnow. *Environ Toxicol Chem* 2020;39:1071–1085. © 2020 SETAC

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INTRODUCTION

Nitrate (NO_3) naturally occurs in surface and groundwater at a level that does not generally cause health problems for aquatic biota. Elevated levels of NO_3 in natural water often result from agricultural activity (including excess application of inorganic nitrogenous fertilizers and manures), from wastewater treatment, and from oxidation of nitrogenous waste products in human and animal excreta, including septic systems (World Health Organization 2011). Sulfate (SO_4) naturally occurs in aquatic environments due to the decomposition of plants, atmospheric deposition, and the weathering of minerals. Human activities, such as mining, can substantially increase geological weathering rates, leading to increased SO_4 concentrations in surface waters (Scofield and Hsieht 1988). Concentrations of SO_4 can also be elevated as the result of discharges from municipal treatment plants, agricultural runoff, and industrial sources, such as tanneries, pulp and paper mills, and textile mills.

National ambient water quality criteria (WQC) in the United States for NO_3 and SO_4 have not been developed for protection of aquatic life. The State of Illinois in the United States has developed state water quality standards for SO_4, but

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only based on acute toxicity data (Illinois Pollution Control Board 2011). Existing toxicity data from acute and chronic NO_3 and SO_4 toxicity tests have recently been reviewed, to facilitate the development of environmental guidance values for NO_3 and SO_4 (Soucek and Dickinson 2012, 2015; Wang et al. 2016). However, more studies are needed to meet the US Environmental Protection Agency (USEPA) guideline for WQC development (Stephan et al. 1985), which requires toxicity data from at least 8 taxonomic groups. The purpose of the present study was to fill existing gaps in the databases for NO_3 and SO_4 by conducting toxicity testing with additional aquatic species.

A previous study indicated that fathead minnow (*Pimephales promelas*) was highly sensitive to sodium sulfate (Na_2SO_4) during the transitional period from embryo to hatching, and the results from a 34-d early life stage toxicity test ranked the fish the most sensitive species in the chronic SO_4 database (Wang et al. 2016). However, additional short-term 7- to 14-d toxicity tests started with newly fertilized eggs in test waters with different ionic compositions at a hardness of 100 mg/L as $CaCO_3$ and indicated that increasing potassium (K) in test waters from 1 mg K/L (used for the earlier 34-d test) to 3 mg K/L substantially reduced the toxicity of Na_2SO_4 (Wang et al. 2016). Further study was needed to evaluate chronic effects of SO_4 on fathead minnow in an early life stage test at a level of K close to the average value in national surface water, such as 3 mg K/L at hardness 100 mg/L as $CaCO_3$ (Wang et al. 2016).

The objectives of the present study were to: 1) determine the acute and/or chronic toxicity of NO_3 to a unionid mussel (fatmucket, *Lampsilis siliquoidea*), a midge (*Chironomus dilutus*), a fish (rainbow trout, *Oncorhynchus mykiss*), and 2 amphibians (gray treefrog, *Hyla versicolor*, and wood frog, *Lithobates sylvaticus*); 2) determine the acute and chronic toxicity of SO_4 to 2 unionid mussels (fatmucket and rainbow mussel, *Villosa iris*) and an amphipod (*Hyalella azteca*); and 3) repeat the 34-d early life stage SO_4 toxicity test with fathead minnow (Wang et al. 2016) but in test water with increased K from 1 to 3 mg/L. In addition, an early life stage SO_4 toxicity test with a commonly tested cold water fish (rainbow trout) was conducted in the same test water used for the repeated early life stage test with the fathead minnow.

For toxicity testing, major ions of Na, NO_3, and SO_4 can only be added in salt form, and thus, the effect of one ion cannot be tested in isolation of the other. Furthermore, whereas early work on major ions concluded that sodium salt toxicity was attributable to the anion (Mount et al. 1997), more recent work has shown that for *Ceriodaphnia dubia* both Na and SO_4 contribute to toxicity (Erickson et al. 2017). The primary salts used to conduct toxicity tests and develop existing regulatory guidelines were sodium nitrate [$NaNO_3$] and Na_2SO_4 (Illinois Pollution Control Board 2011; Canadian Council of Ministers of the Environment 2012; British Columbia Ministry of Environment 2013). However, the guidelines focused specifically on NO_3 or SO_4 concentration, rather than the combination of ions. Therefore, to facilitate comparison with other published data and regulatory guidelines, the results from the present study are expressed as NO_3 (for $NaNO_3$) and SO_4 (for Na_2SO_4) concentrations, but this should not be taken as an assertion that these are the only appropriate exposure metrics.

MATERIALS AND METHODS

All tests were conducted at the US Geological Survey, Columbia Environmental Research Center (CERC; Columbia, MO, USA), except the amphipod tests, which were completed at the Illinois Natural History Survey (INHS; Urbana-Champaign, IL, USA).

Test organisms

Mussels. Gravid female fatmucket and rainbow mussels were collected in early spring from the Silver Fork of Perche Creek (Boone County, MO, USA) and Indian Creek (Tazewell, VA, USA), respectively. The female mussels were transferred to Missouri State University (Springfield, MO, USA) for juvenile mussel metamorphosis. The glochidia isolated from at least 3 fatmucket and 7 rainbow mussels were pooled and placed on largemouth bass (*Micropterus salmoides*) for fatmucket metamorphosis and on rock bass (*Ambloplites rupestris*) for rainbow mussel metamorphosis. Metamorphosed juvenile mussels collected from the host fish during the 2-d peak drop-off were shipped overnight to the CERC. A subset of the newly metamorphosed mussels was used for acute toxicity testing after at least 48-h acclimation to test water (i.e., control water; details in the *Preparation of test water* section) and test temperature of 23 °C. The remaining juveniles were cultured for 2 to 5 wk at CERC under a flow-through autofeeding system (Wang et al. 2018a) in test water and temperature before use in chronic exposure. Additional details on mussel metamorphosis at the Missouri State University and juvenile culture at the CERC are described in previous publications (Wang et al. 2018b).

Midges and fish. First, 7-d-old larval midges and embryonic fathead minnow were obtained from laboratory cultures at the CERC. Eyed-stage embryos of rainbow trout were obtained from the Ennis National Fish Hatchery (Ennis, MT, USA) for toxicity testing and culturing larval and juvenile rainbow trout for toxicity testing. All test species were cultured in test water and temperatures (23 °C for midges, 25 °C for fathead minnows, and 12 °C for rainbow trout).

Amphipods. The strain of the amphipod used for the present study was genetically identified as the "US Lab strain" by Major et al. (2013), and complete cytochrome oxidase subunit 1 (COI) sequences can be found at GenBank accession nos. JX446307 and JX446308 (National Center for Biotechnology Information 2015). Amphipods were cultured at INHS in test water at a test temperature of 23 °C. Twenty-five to 30 individuals were held in 1-L beakers with 1000 mL of water. Nylon screen (1-mm mesh size, 44% open area; ELKO Filtering) was provided as the substrate. At any given time 3 to 5 beakers were maintained. Organisms in each beaker were fed daily 6.0 mg of dry, ground and sieved (<250 µm) TetraMin® (TetraWerke), and 5.0 mg dry weight of mixed diatom solution

prepared as described in Soucek et al. (2013). Culture water was changed and screens were cleaned twice weekly. After collection, young were transferred to an environmental chamber for holding until testing. During this holding period, the amphipods (~1 wk old) in each beaker were fed 3.0 mg of dry, ground and sieved (<250 μm) Tetramin, and 5.0 mg dry weight of mixed diatom solution daily.

Amphibians. Adult gray treefrogs were collected from the Baskett Wildlife Research Area (Ashland, MO, USA). Three breeding pairs were transported to CERC and held overnight in inclined polypropylene tubs (63 × 41 × 17 cm) partially filled with test water (CERC well water; hardness ~300 mg/L as $CaCO_3$, pH 8.2) at the test temperature of 25 °C. Oviposited eggs were maintained as separate clutches until testing. For acute tests, eggs were maintained through hatch and subsequently to Gosner stage 25 (the first free-swimming, exogenous-feeding stage; Gosner 1960), with 50% water changes daily. Prior to being stocked in the test, tadpoles were fed ground TetraMin fish flakes (Tetra) ad libitum for 3 d. Tadpoles were approximately 6 d post hatch (dph) at the beginning of the acute tests. Wood frog egg masses were collected from a temporary pond on the Daniel Boone Conservation Area (Warren County, MO, USA). Eggs were transported to CERC in polypropylene containers of source water at the collection temperature (12 °C). Eggs were maintained and tadpoles were fed as just described for gray treefrogs, except that wood frogs were acclimated to test water over 3 d. and were maintained at the test temperature of 12 °C. Acute tests began when tadpoles reached Gosner stage 25 (~6 dph).

Ambient laboratory light (~500 lux) was used with 16:8-h light:dark photoperiod during the culture and toxicity testing at CERC, except that the trout embryos were incubated in the dark, and newly hatched trout were held at a low-light intensity of 70 lux for the first 10 d of the exposures, and afterwards, the light intensity was increased to 500 lux based on guidance provided in Environment Canada (1998). Ambient laboratory light (~160 lux) was used with a 16:8-h light:dark photoperiod during the culture and toxicity testing with amphipods at the INHS.

Preparation of test water

The test water (control water) for species tested at CERC was prepared by diluting CERC well water (hardness ~300 mg/L as $CaCO_3$) with deionized water to a hardness of 100 mg/L as $CaCO_3$, except CERC undiluted well water was used for the amphibian tests. For the tests to evaluate the influence of K on chronic SO_4 toxicity to fish, KCl (99.7% purity; Fisher Scientific) was added into the diluted well water to increase the concentration of K from 1.0 to 3.0 mg/L. The addition of KCl to increase K resulted in a minimal increase in Cl. The test water for the amphipods tested at INHS was a reconstituted water with a nominal hardness of 100 mg/L as $CaCO_3$ (hereafter termed Duluth 100 hard water), prepared according to a formula developed at the USEPA laboratory in Duluth, Minnesota (D.R. Mount, personal communication). This water recipe was designed with the goal of better mimicking the chemistry of "typical" North American freshwaters relative to other commonly used reconstituted waters, and the water was made by adding the following concentrations of salts to deionized water: 10 mg/L $KHCO_3$, 125 mg/L $NaHCO_3$, 38 mg/L $MgSO_4$ (anhydrous), 40 mg/L $CaSO_4$ (anhydrous), 43 mg/L $CaCl_2$ (anhydrous), and 0.05 mg/L NaBr.

Preparation of test solutions

American Chemical Society–grade $NaNO_3$, (99% purity; Sigma-Aldrich) and Na_2SO_4 (99% purity; Sigma-Aldrich) were used for test solution preparation at CERC. For testing at INHS, Na_2SO_4 (99% purity, Fisher Scientific, Itasca, IL) was used. Five exposure concentrations plus a control were tested, except for an acute test with gray treefrogs tested in 6 concentrations plus a control. For acute toxicity testing under static or static-renewal conditions, a solution of the highest exposure concentration was prepared 12 to 24 h before the start of a test by adding a certain amount of $NaNO_3$ or Na_2SO_4 into 2 L of control water in a glass jar for invertebrate tests or up to 25 L of control water in a large container for fish and amphibian tests. A portion of the solution was then used for 50% dilutions to create other solutions of lower exposure concentrations, with an exception for a few tests. A 30% dilution series was used in the NO_3 exposure with the gray treefrog and in the SO_4 exposures with the 2 mussel species.

The control water and solutions were held in water baths at test temperature for use at the beginning of a test and for water renewal. For chronic flow-through toxicity testing, an intermittent flow-through proportional diluter system (Wang et al. 2007, 2016) modified from Mount and Brungs (1967) was used. Each diluter with one test species created and delivered 5 test concentrations with a 50% dilution series, plus a control. A stock of test solution was delivered with each cycle of the diluter by a Hamilton® syringe pump. For the chronic SO_4 test, the solution for the highest concentration was prepared in 2 2000-L recirculating tanks. (Tank A was used to deliver the high concentration to the diluter, and tank B was used to prepare a new batch of the solution once every week or every other week depending on the remaining water in tank A).

Test concentrations were chosen based on results of previous studies with the same species used in the present study (Buhl and Hamilton 2000; Soucek 2007; US Environmental Protection Agency 2010; Elphick et al. 2011; Wang et al. 2016, 2017). When a prediction of acute toxicity of NO_3 or SO_4 was not available for species tested in the present study, an initial range-finding test was first conducted with a limited number of organisms (5–10) and replicates (1 or 2) in a control and 5 concentrations of the test chemical that differed by a dilution factor of up to 10. Generally, no chronic effect concentrations for NO_3 or SO_4 were available for the species tested in the present study. Nominal chronic exposure concentrations were chosen based on acute effect concentrations for the test species if available or based on chronic effect concentrations for sensitive species reported in previous

studies with chronic $NaNO_3$ exposures (*Salvelinus namaycush* in McGurk et al. 2006, *C. dubia* in Scott and Crunkilton 2000, *P. promelas* in US Environmental Protection Agency 2010, *Neocloeon triangulifer* in Soucek and Dickinson 2015, *H. azteca* in Soucek and Dickinson 2016, and *Pseudacris regilla* in Schuytema and Nebeker 1999a, 1999b) and with chronic Na_2SO_4 exposures (*P. regilla* in Elphick et al. 2011 and *P. promelas* and *Lampsilis abrupta* in Wang et al. 2016). Nominal test concentrations of test solutions were calculated based on NO_3-N and SO_4, except for the NO_3 toxicity tests with rainbow trout, for which the nominal concentrations were calculated mistakenly based on NO_3 (rather than NO_3-N) during preparation of the test solutions, resulting in actual test concentrations 4-fold lower than those bracketing effect concentrations from previous studies.

Acute exposures with different species

Acute toxicity tests were conducted with different species in basic accordance with standard methods (ASTM International 2019a, 2019b, 2019c). At the beginning of the mussel and midge tests, 5 juvenile mussels were impartially transferred into each of 4 replicate 50-mL glass beakers containing 30 mL of test solution, and one midge was transferred into each of 10 replicate 50-mL glass beakers containing 30 mL of test solution and 5 mL of silica sand (~100- to 400-μm particles; Granusil #5010; Unimin), which had been held in control water for 24 h prior to testing. All test beakers in each test with a species were held in a plastic holding container (30 × 18 × 10 cm) with a cover to reduce evaporation. The containers were placed in a water bath at 23 ± 1 °C. At the beginning of the fish test, 5 rainbow trout were transferred into each of 4 replicate 4-L glass jars containing 2.5 L of test solution, keeping fish loadings below the maximum loading rate of 0.8 g/L wet weight recommended in ASTM International (2019a). The jars were held in a water bath at 12 ± 1 °C. At the beginning of the amphibian tests, 10 gray treefrog or wood frog larvae were transferred into each of 4 replicate 3-L jars containing 2 L of test solution. Jars were held in a water bath at either 25 ± 1 °C (gray treefrogs) or 12 ± 1 °C (wood frogs), reflecting typical ambient conditions during larval development for each species. At the beginning of the amphipod test, 5 amphipods were impartially transferred into each of 4 replicate 50-mL glass beakers containing 30 mL of test solution at 23 ± 1 °C. Squares of nylon screen (~2 × 2 cm) were provided as substrate.

Test durations were 48 h for the midges and 96 h for the other species. Test solutions were not renewed in exposures with midges, amphipods, and amphibians, but were renewed at 48 h in exposures with mussels and trout by replacing approximately 70% of the water in each test beaker with fresh test solution. Test organisms were not fed, except for amphibians. The amphibians in each jar were fed a light dusting of ground TetraMin fish flakes at 48 h to avoid confounding symptoms of toxicity with starvation of the early larvae (Hecnar 1995). The numbers of immobilized mussels (no foot movement within 5 min), midges, and amphipods (lack of response to gentle prodding) were recorded at the end of exposures. Immobilized and dead fish and amphibians were recorded daily, and dead fish and amphibians were removed.

Chronic exposures with mussels and midges

Chronic 28-d toxicity tests with mussels and 10-d toxicity tests with midges were conducted in the flow-through diluters following standard methods (ASTM International 2019b, 2019c). At the start of a test, 10 organisms were impartially transferred into each of 300-mL replicate glass beakers. Each beaker contained 10 mL of sand substrate (~100- to 400-μm particles; Granusil #5010) and was equipped with a 2.5-cm hole in the side that had been covered with a 50-mesh (279-μm width opening) stainless-steel screen to allow the solution to flow through and hold 200 mL of water. For the NO_3 toxicity test with the mussel (fatmucket) and midge, each species was tested in a diluter with 8 replicate beakers/exposure concentration. The diluter provided 125 mL of test solution to each replicate beaker every 4 h (~4 water volume additions/d). For the SO_4 toxicity tests, the 2 mussel species (fatmucket and rainbow mussel) were tested side by side in a diluter; each species had 4 replicates. The diluter provided 125 mL of test solution to each replicate beaker every 2 h (~8 water volume additions/d). Replicate beakers in each diluter were held in a water bath at 23 ± 1 °C. Mussels were fed 2 mL of algal mixture (~510 nL cell volume/L) delivered by a peristaltic pump (Dosing Pump; Jiyang Aquarium Equipment) automatically into each replicate beaker with each cycling of diluter (Wang et al. 2018a). The algal mixture was prepared daily by adding 1 mL of a commercial nonviable microalgal *Nannochloropsis* concentrate (Nanno 3600™) and 2 mL of a mix of 6 microalgae (Shellfish Diet 1800™; Reed Mariculture) into 1.8 L of test water. Test beakers and sand in the mussel tests were replaced on test day 14, and mussels in each beaker were first rinsed into a 200-mL glass dish with test solution from the beaker for survival determination. Mussels with a gaped shell containing swollen or decomposed tissue or with an empty shell were classified as dead and removed from replicate beakers. Surviving mussels were transferred into new beakers containing new sand. The midges in each replicate were fed 1.0 mL of TetraMin mixture (4.0 mg of dry solids) twice daily from test days 0 to 3, and 1.5 mL of TetraMin mixture (6.0 mg of dry solids) once daily starting on test day 4. At the end of the exposure, surviving mussels and midges isolated from each replicate were counted. Dry weight of the surviving mussels/replicate (biomass) was determined by drying the organisms at 60 °C for 48 h, and ash-free dry weight of midges/replicate (biomass) was determined after ashing at 550 °C for 2 h and measuring to the nearest 0.001 mg.

Chronic exposure with amphipods

A chronic 42-d, static-renewal toxicity test was performed using recommendations detailed in the USEPA sediment toxicity testing guidelines (US Environmental Protection Agency 2000), but with modifications. Six treatments including a control were comprised of a 50% dilution series. Test chambers were 300-mL,

high-form glass beakers with 200 mL of test water added to each beaker. Approximately 3.5- × 4.0-cm pieces of nylon screen (1-mm mesh size, 44% open area; ELKO Filtering) were provided to each beaker as substrate. Tests were performed at 23 ± 1 °C without aeration. Organisms were 7 to 9 d old at the beginning of the tests, and 10 individuals were added to each of 5 replicate test chambers/treatment. Complete water renewals were performed 3 times weekly (Mondays, Wednesdays, and Fridays), with test organisms being transferred to clean weigh-boats containing test water while test chambers were cleaned. Substrates were replaced once every week. During the test, organisms were fed once daily with a mixed diatom solution and ground and sieved (<250 µm) TetraMin. Diatoms were added to beakers at a constant rate of 1 mg dry weight throughout the test. TetraMin feeding rate was as follows: 1 mg dry weight during week 1, 1.25 mg during weeks 2 and 3, and 2.5 mg daily during weeks 4 through 6. After the first appearance of mating pairs, the contents of each test chamber were carefully searched for young, which were counted, on water renewal days. On test day 42, adult amphipods were placed in a small amount of 95% ethanol so they could be examined under a dissecting scope to determine sex (males with an enlarged and modified second gnathopod). The adult samples were then immediately transferred to aluminum weigh pans and dried in an oven (60–70 °C) for 48 h before they were weighed to the nearest 0.001 mg. Based on subsamples of organisms used to initiate the tests, average starting weights for the 42-d tests were 0.022 mg dry weight/individual. Endpoints included percentage of survival, dry weight (average dry wt/individual), biomass (total dry wt of surviving adults in a replicate), and number of young/surviving female.

Chronic exposures with fish

Early life stage toxicity tests were conducted starting with <48-h-old embryos of fathead minnow and eyed-stage embryos of rainbow trout (SO_4 exposure) or 1-dph rainbow trout (NO_3 exposure) in 2 flow-through diluters following standard methods (ASTM International 2019d). The minnow test was conducted at 25 ± 1 °C for 34 d (28 d beyond the mean day to hatch of the controls), and the trout tests were conducted at 12 ± 1 °C for >40 d (30 d after swimming up of the controls). Each diluter with a test species provided 500 mL of test solution into each of 4 replicate chambers (containing 7 L of water) every hour (1.7 volume replacement). The water delivery frequency was increased to a cycling of 500 mL of water every 30 min to ensure the fish loading never exceeded the maximum loading rates of 5 g/L at any time and 0.5 g/L of solution passing through the chamber in 24-h recommended in ASTM International (2019d).

Because of the uncertainty of fertilization success with minnow eggs, large numbers of embryos were used for each test chamber and then thinned to the desired number of embryos on test day 2 as recommended by ASTM International (2019d). Specifically, at the beginning of the test, 30 <48-h-old embryos were impartially transferred into an incubation cup (1000 mL with 40-mesh stainless-steel screen) suspended in each test chamber. Test solutions flowed directly into the cups. On test day 2, the embryos were examined by placing the cups under a dissecting scope, and dead embryos and embryos with fungal growth (<10% embryos/replicate) were counted and discarded. Embryos remaining on test day 2 were impartially thinned to 15. The numbers of fish hatched in each replicate chamber were recorded daily on test days 3 to 6. Dead fish were recorded and removed during the daily observation. On test day 6 when all fish hatched, live fish were released into the surrounding test chamber for the remainder of the 34-d exposure. The fish were fed <24-h-old brine shrimp nauplii 3 times daily at an interval of 4 h during weekdays and 2 times daily at an interval of 6 h on the weekends. Sufficient numbers of nauplii were provided to ensure that some nauplii remained alive in the test chamber for several hours after each feeding. Before the nauplii were added to the replicate chambers, concentrated nauplii in a 120-mesh nylon net were gently rinsed with control water. Uneaten brine shrimp, dead fish, and other debris were removed by siphoning with a small-bore glass tube prior to the first feeding of the day. A count of surviving fish was made once weekly. At the end of the test, surviving fish were counted and euthanized with an overdose of 200 mg/L tricaine methanesulfonate (Western Chemical). The study complied with all applicable sections of the Final Rules of the Animal Welfare Act regulations (9 CFR) and with all CERC guidelines for the humane treatment of the fish during culture and experimentation. Total length of individual fish was measured to the nearest 0.1 mm, and dry weight of fish/replicate was determined by drying fish at 60 °C for 48 h and measuring to the nearest 0.001 mg.

At the beginning of the SO_4 test with rainbow trout, 15 eyed embryos were impartially transferred into each incubation cup. The numbers of the trout hatched in each replicate chamber were recorded daily. The hatch started on test day 4 across all treatments. More than 90% of the trout hatched on test day 7, and all had hatched on test day 8, except for one trout in the highest treatment that hatched on test day 11. Hatched fish were released into the surrounding test chambers as described previously for the minnow test in this section. At the beginning of the NO_3 test with rainbow trout, 10 1-dph fish were transferred impartially into each replicate chamber. The numbers of hatched and swim-up fish in each test were counted daily. Food was provided before the onset of exogenous feeding. The exogenous feeding was confirmed by the observation of feeding activity and feces. The fish were fed <24-h-old brine shrimp ad libitum 3 times daily at an interval of 4 h during the weekdays and 2 times daily at an interval of 6 h during the weekend. Sufficient numbers of nauplii were provided to ensure that some remained alive in the test chamber for at least 2 h. In addition, a minimal amount of live oligochaetes was added to each chamber to determine the readiness of fish to consume oligochaetes. The oligochaetes initially were cut into pieces (~5-mm length) to facilitate initial feeding of larval fish. When the fish were large enough to begin feeding on oligochaetes, more oligochaetes were added, and the amount of brine shrimp was reduced gradually until the fish were fed only oligochaetes twice daily in excess (food available for at least 4 h after each feeding). Before daily feeding in the morning, uneaten food and debris were removed by siphoning, and dead fish were removed and recorded. Fish were not fed for 24 h

before sampling for growth measurements at the end of an exposure. At the end of the exposures, surviving fish in each replicate chamber were counted, euthanized with an overdose of tricaine methanesulfonate, and measured individually in total length to the nearest 0.1 mm. After length measurements, dry weights of fish/replicate were determined by drying fish at 60 °C for 48 h measured to the nearest 0.001 mg.

Chronic exposure with amphibians

A chronic NO_3 toxicity test was conducted starting with gray treefrog embryos <24 h post fertilization (Gosner stage 9–10, late cleavage to dorsal lip formation; Gosner 1960), based on standard methods (ASTM International 2019a, 2019d). The amphibian test was conducted in a diluter at 25 ± 1 °C for the full larval period (52 d, at which time a mean of 98% of control animals had metamorphosed). The diluter provided 500 mL of test solution into each of 8 replicate 7-L chambers every hour (1.7 volume replacement). At the beginning of the test, 4 eggs from each of 3 clutches ($n = 12$/chamber) were stocked in incubation cups that held the eggs suspended approximately 5.5 cm below the water's surface while allowing free exchange with the larger volume of water in each experimental chamber. The incubation cups remained in place until all viable eggs had hatched (day 4), at which time larvae were released into the 7-L chambers for the duration of the test. Larvae in each chamber were fed ground TetraMin fish flakes daily. The mass of food was adjusted as larvae grew to ensure that uneaten food remained for at least 1 h after feeding, but not more than 4 h to avoid excessive fouling of the water. Overflow screens on each chamber were cleaned daily, and waste was siphoned from chambers twice weekly or as needed. Chambers were checked daily for immobilized larvae, and any dead amphibians were removed. At the onset of metamorphosis (stage 42, Gosner 1960; defined as the emergence of at least one forelimb), metamorphs were removed from experimental chambers to prevent drowning and housed individually in plastic containers until metamorphosis was complete (stage 46; defined as complete tail resorption). At that point, each metamorph was blotted dry and individually weighed to the nearest 0.1 mg. Endpoints for the chronic test included percentage of survival (number of metamorphs plus number of remaining tadpoles in each chamber divided by the number of eggs originally stocked), percentage of metamorphosis (number of metamorphs from each chamber divided by the number of eggs originally stocked), time to metamorphosis (mean number of days from stocking to completion of metamorphosis for metamorphs in each chamber), individual weight at metamorphosis (mean wet wt at the completion of metamorphosis for individual metamorphs in each chamber), and biomass (total wet wt of all metamorphs from a given chamber).

Water quality and chemical analyses

For the tests conducted at CERC, dissolved oxygen, pH, conductivity, hardness, alkalinity, and ammonia (generally only during a chronic test) were measured using standard methods (Eaton et al. 2005) on composite water samples collected from the replicates in the control, medium, and high exposure concentrations at the beginning and the end of acute tests and once weekly or biweekly during chronic tests. Water temperature in test chambers in each water bath was monitored daily. Composite water samples for NO_3 and SO_4 measurement were collected from each exposure concentration at the beginning and the end of acute tests and once weekly or biweekly during chronic tests. The NO_3 and SO_4 were analyzed with a Hach spectrophotometer (model DR 2800) except that a Technicon Auto-Analyzer II following Standard Method 4500-NO_3 F (Eaton et al. 2005) was used for NO_3 analyses in amphibian tests. Conductivity was measured periodically in chronic exposures to monitor exposure concentrations. Composite water samples for analysis of major cations (calcium, K, magnesium, and sodium) and major anions (Cl and SO_4) were collected from the control and medium concentrations at least once during acute tests and once every 2 wk in chronic exposures. The cations were analyzed with inductively coupled plasma–mass spectrometry (ELAN DRC-e; PerkinElmer) using a method similar to USEPA method 6020B (US Environmental Protection Agency 2014). The anions were quantified by ion chromatography (ICS-1100; Dionex) using a method similar to USEPA method 9056A (US Environmental Protection Agency 2007). At INHS, water quality and chemical sampling and analyses were similar to those just described for the tests conducted at CERC, except that Cl (background levels in dilution water) and SO_4 concentrations for amphipod tests were measured at the Illinois State Water Survey analytical laboratory.

Analyses of tested chemicals were performed following CERC and INHS internal standard operating procedures and quality assurance/quality control protocols. Established laboratory quality assurance/quality control procedures and sample types (i.e., second-source calibration verification, laboratory spikes, duplicates, reference/laboratory control materials) were used to verify instrument performance, accuracy, and precision throughout the analyses. These established procedures were in place to ensure method performance and instrument suitability. Results from each laboratory underwent data quality review prior to use in the present study.

Data analyses

Biological endpoints were analyzed using measured exposure concentrations. Acute 50% effect concentrations (EC50s) were calculated based on mortality plus immobility. Chronic 10 and 20% effect concentrations (EC10s and EC20s) were estimated for survival and sublethal endpoints using the Toxicity Relationship Analysis Program (TRAP; Erickson 2015). In general, the tolerance distribution analysis with a Gaussian (normal) distribution model was used for survival data analyses, and the nonlinear regression analysis with threshold sigmoid regression was used for sublethal endpoint data analyses. The exposure concentrations were log-transformed, and the response of each replicate was used for the calculation. When the data did not meet the requirements of the TRAP (at least 2 partial responses), either a Spearman–Karber or trimmed Spearman–Karber method was used following the flowchart for acute EC50 determination (US Environmental Protection Agency 2002a), and a linear interpolation method

(US Environmental Protection Agency 2002b) was used to estimate chronic EC20s using TOXSTAT® software (Ver 3.5, Western EcoSystems Technology). In addition, a maximum acceptable toxicant concentration (MATC) was calculated as the geometric mean of the no-observed-effect concentration (NOEC) and the lowest-observed-effect concentration (LOEC). The NOEC and LOEC were determined by Williams' test (or by Steel's many-one rank test if data were not normally distributed or did not have equal variances) using TOXSTAT software. When there are survival differences among treatments, the growth analysis can be a biased measure because toxicants may selectively kill smaller organisms and thus increase the average length or weight of surviving organisms (Gelber et al. 1985). In addition, when there are survival differences, the number of surviving organisms in individual test chambers may differ widely, which can limit the validity of the test statistics. To focus on effects on growth that occurred at exposure concentrations less than those affecting survival, the growth (length or weight) and biomass data from the exposure concentrations that exhibited a significant difference from the control for survival were excluded from further LOEC determination (US Environmental Protection Agency 2002b). The level of statistical significance was set at $\alpha = 0.05$.

To compare the relative sensitivity among all freshwater organisms tested, toxicity databases were compiled based on the results of the present study and published data from $NaNO_3$ and Na_2SO_4 toxicity tests with North American native species. The published data were obtained mainly by reviewing the NO_3 toxicity database used for developing the 2010 draft Minnesota water quality standards for NO_3 (Monson 2010) and a recently compiled SO_4 toxicity database of Wang et al. (2016) with new data found through the USEPA ECOTOX website (US Environmental Protection Agency 2019). The lethal concentration (LC)/EC50s were used for acute effect concentrations, and LC/EC20s were preferred for chronic effect concentrations. When LC/EC20s were not reported in the original papers, then 25% inhibition concentrations (IC25s) or MATCs were used for chronic effect concentrations. The databases included only data that met test acceptability criteria, which were based on the guidelines for deriving WQC (Stephan et al. 1985) and standard test methods (ASTM International 2019a, 2019b, 2019c, 2019d), including control survival ≥90% in acute exposures and ≥80% for chronic exposures; acute test durations were 48 h for daphnids and 96 h for others; chronic test durations were 6 to 8 d in reproduction tests with *C. dubia* and 21 d in reproduction tests with *Daphnia* spp., ≥7 d in early life stage tests with larval midges, mayflies, and amphibians, ≥28 d in mussel and amphipod tests, and ≥28 dph (≥30 d post swim-up for salmonids) in the early life-stage tests starting with embryos or newly hatched fish <48 h old.

RESULTS AND DISCUSSION

Water quality and chemical analyses

Detailed water-quality results are presented in the Supplemental Data, Tables S1 and S2. Water-quality characteristics were consistent among different treatments in all acute and chronic toxicity tests conducted within a test water. In tests using the CERC diluted well water, mean water-quality measurements ranged from pH 7.8 to 8.4, hardness from 102 to 109 mg/L as $CaCO_3$, alkalinity from 91 to 100 mg/L as $CaCO_3$, Ca from 25 to 27 mg/L, K from 0.9 to 1.2 mg/L, Mg from 8.7 to 9.6 mg/L, Na from 9.8 to 15 mg/L, Cl from 11 to 13 mg/L, and SO_4 from 16 to 21 mg/L, with the exception of a mean concentration of K of 3.0 mg/L in the fathead minnow test and 2.8 mg/L in the trout test when using the CERC diluted well water with an addition of K to increase the concentration of K from 1 to 3 mg/L. In the amphibian tests using undiluted CERC well water, mean water-quality measurements ranged from pH 8.2, hardness from 287 to 305 mg/L as $CaCO_3$, and alkalinity from 250 to 262 mg/L as $CaCO_3$. The major cation and anions in the CERC well water for the amphibian tests were not measured. The concentrations of the major ions in the well water were reported as Ca 70 mg/L, K 3.0 mg/L, Mg 30 mg/L, Na 26 mg/L, Cl 20 mg/L, and SO_4 53 mg/L (Wang et al. 2019). In the amphipod tests with the Duluth 100 hard water, mean water-quality measurements ranged from pH 8.1 to 8.3, hardness from 101 to 105 mg/L as $CaCO_3$, alkalinity from 81 to 82 mg/L as $CaCO_3$, Cl from 25 to 28 mg/L, and SO_4 from 54 to 59 mg/L. The major cations in the Duluth 100 hard water were not measured; the nominal concentrations were Ca 27 mg/L, K 3.9 mg/L, Mg 7.7 mg/L, and Na 34 mg/L. Across all tests with different species, the mean concentration of dissolved oxygen was high (≥7.1 mg/L), and mean concentrations of total ammonia nitrogen (TAN) were low (≤0.06 mg TAN/L in tests with mussels, which are highly sensitive to ammonia; ≤0.21 mg TAN/L in tests with other test species).

Conductivities in each test varied with test treatments due to the addition of the toxicant ($NaNO_3$ or Na_2SO_4) and were consistent with the increasing exposure concentrations (Supplemental Data, Tables S3–S13). Mean measured exposure concentrations were close to their nominal concentrations, and the difference between the measured and nominal was typically <10% (Supplemental Data, Tables S3–S13).

Acute NO_3 toxicity to the mussel, midge, fish, and amphibian

Control survival of all test species in acute exposures ranged from 90 to 100% (Table 1) and met the acute test acceptability criterion of ≥90% control survival (ASTM International 2019a, 2019b). Acute EC50s for the 5 species ranged from 189 to >883 mg NO_3-N/L (Table 1). The midge was the most sensitive species. The mussel, with an EC50 of 665 mg NO_3-N/L, had similar sensitivity to the 2 species of amphibians (601 mg NO_3-N/L for gray treefrog and 694 mg NO_3-N/L for wood frog). The trout was the least sensitive species among the 5 test species.

Although Baker et al. (2017) reported that increasing ionic strength reduced the toxicity of NO_3 to several species, their test waters had varying concentrations of all major ions, making it difficult to determine which ones played the strongest role in regulating NO_3 toxicity. Furthermore, although Soucek et al. (2015) and Soucek and Dickinson (2016) observed a strong

TABLE 1: Acute and chronic effect concentrations (95% confidence limits in parentheses) for nitrate (mg NO_3-N/L) in sodium nitrate exposures with fatmucket (*Lampsilis siliquoidea*), midge (*Chironomus dilutus*), rainbow trout (*Oncorhynchus mykiss*), gray treefrog (*Hyla versicolor*), and wood frog (*Lithobates sylvaticus*)

Species	Test (test duration)	Age of organism at test start	Control survival (%)	Acute EC50 (mg NO_3-N/L)	Chronic LC20 or EC20 (mg NO_3-N/L)			
					Survival	Weight	Biomass	Metamorphosis
Fatmucket								
	Acute (4 d)	~6-d juveniles	100	665 (no CL)[a]	—[b]	—	—	—
	Chronic (28 d)	~14-d juveniles	100	—	>72[c]	17 (10–30)	18 (10–30)	—
Midge								
	Acute (2 d)	7-d larvae	90	189 (170–210)	—	—	—	—
	Chronic (10 d)	7-d larvae	96	—	93 (49–96)	NE[d]	9.6 (5.6–16)	—
Rainbow trout								
	Acute (4 d)	30-dph juveniles	100	>883	—	—	—	—
	Chronic (42 d)	1-dph juveniles	100	—	>38[e]	>38[e]	>38[e]	—
Gray treefrog								
	Acute (4 d)	~6-dph larvae	100	601 (536–673)	—	—	—	—
	Chronic (52 d)	<24-h embryos	94	—	>111	>111	>111	47 (33–92)
Wood frog								
	Acute (4 d)	~6-dph larvae	100	694 (629–765)	—	—	—	—

[a]No CL could be calculated because of no partial effect (see Supplemental Data, Table S3).
[b]Not applicable.
[c]A greater-than value was reported when no exposure concentration caused an effect of >40% in acute exposures and >15% in chronic exposures relative to the control.
[d]An EC20 for weight was not estimated because of the uncertainty of the density-dependent growth effect (see Supplemental Data, Table S6).
[e]This greater-than value should be used with caution because an actual effect concentration could be much greater than 38 mg NO_3-N/L (see text).
EC20 or EC50 = 20 or 50% effect concentration; LC20 = 20% lethal concentration; CL = confidence limit; dph = day post hatch; NE = not estimated.

influence of chloride on NO_3 toxicity to the US Lab genetic strain of the amphipod (*H. azteca*), this influence was observed neither in tests with the cladoceran (*C. dubia*) nor in another strain of *H. azteca* (Burlington strain). Because of the lack of consistent evidence of a specific background ionic effect on NO_3 toxicity, we compared acute and chronic effect levels as reported rather than normalizing for a particular background ion.

The EC50 of 665 mg NO3-N/L for fatmucket in the present study was greater than a previously published EC50 of 357 mg NO_3-N/L for this species (Soucek and Dickinson 2012). Another previously tested native mussel (*Megalonaias nervosa*) and 2 European unionid mussels (*Anondonta anatine* and *Unio crassus*) were less sensitive than the fatmucket, with EC50s ranging from 922 to 1272 mg NO_3-N/L (Douda 2010; Soucek and Dickinson 2012). The midge EC50 of 189 mg NO_3-N/L from the present study was similar to a previously published EC50 of 278 mg NO_3-N/L for this species (US Environmental Protection Agency 2010). In our rainbow trout test, the exposure concentrations were prepared mistakenly based on NO_3 rather than NO_3-N, as described previously in the *Preparation of test solutions* section, and the concentrations of up to 883 mg NO_3-N/L were not high enough to cause mortality. Two previous studies reported similar LC50s (1958 and 1658 mg NO_3-N/L) for rainbow trout (Buhl and Hamilton 2000; Baker et al. 2017).

Many previous studies of NO_3 toxicity to amphibians have used ammonium nitrate (NH_4NO_3) as an NO_3 source. Although these studies provide useful information on the toxicity of common fertilizers, a portion of the toxicity attributed to NO_3 may have been caused by NH_3 (Johansson et al. 2001; Camargo et al. 2005). Among studies using $NaNO_3$ as an NO_3 source, the only acute LC50 values for native North American amphibian species are for the Pacific treefrog (*P. regilla*; Schuytema and Nebeker 1999a, 1999b). The EC50 values we reported for gray treefrog and wood frog tadpoles (601 and 694 mg NO_3-N/L, respectively) are very similar to the LC50 reported for Pacific treefrogs at the embryo stage (643 mg NO_3-N/L) but lower than that reported for the tadpole stage (1750 mg NO_3-N/L; Schuytema and Nebeker 1999a, 1999b).

Chronic NO_3 toxicity to the mussel, midge, fish, and amphibian

Mean control survival of the mussel, rainbow trout, and gray treefrog in chronic exposures ranged from 94 to 100% (Table 1) and met the chronic test acceptability criterion of ≥80% control survival (ASTM International 2019b, 2019d). Mean control survival of the midge was 96%, with a mean ash-free dry weight of 1.74 mg at test day 10 (Supplemental Data, Table S6) and met the test acceptability criteria for the control of >70% survival and >0.48 mg ash-free dry weight/organism for a 10-d midge test (ASTM International 2019c).

The dry weight and biomass of the mussel were significantly reduced in the 2 high treatments (LOEC 34 mg NO_3-N/L; Supplemental Data, Table S5), and the dry weight and biomass of the midge were significantly reduced in all treatments compared with the control (LOEC 5.7 mg NO_3-N/L; Supplemental Data, Table S6). Because the midge dry weight and biomass at the lowest exposure concentration were significantly reduced (by 18 and 21%, respectively) relative to the controls, it is uncertain that the lowest exposure concentration was a true LOEC. Thus, further study with an additional exposure concentration below the

LOEC may be needed to confirm the effect concentration. The mean survival and growth of rainbow trout in all 5 treatments of up to 38 mg NO_3-N/L were not significantly different from the controls, and no effect concentrations could be calculated (Supplemental Data, Table S7). The exposure concentrations for the trout test were prepared mistakenly based on NO_3 rather than NO_3-N as described previously in the *Preparation of test solutions* section, and therefore, an actual effect concentration would be much greater than 38 mg NO_3-N/L. In the gray treefrog test, significant reductions in metamorphosis were found in the 2 high treatments (LOEC 52 mg NO_3-N/L; Supplemental Data, Table S8). Chronic effect concentrations based on the most sensitive endpoint were 17 mg NO_3-N/L for the mussel, 9.6 mg NO_3-N/L for the midge, and 47 mg NO_3-N/L for the treefrog (Table 1). Therefore, the midge was the most sensitive species and the rainbow trout was likely the least sensitive species among the 4 tested species in chronic NO_3 exposures. In contrast to the findings in acute NO_3 exposure, however, the mussel was more sensitive than the amphibian in chronic exposure.

Of the 4 test species in our chronic NO_3 tests, only the midge and the gray treefrog were tested in a previous study. Baker et al. (2017) reported that 25% inhibition concentrations (IC25s), ranged from approximately 49 to 178 mg NO_3-N/L based on midge growth, in 10-d exposures using 3 different waters with varying ionic strengths, including a moderately hard reconstituted water that had water quality characteristics similar to the test water we used in the present midge test. To better compare their data with ours, we calculated the EC20 for biomass based on the mean survival and dry weight provided in the Baker et al. (2017) Supplemental Data tables. The estimated EC20 of 91 (76–108) NO_3-N/L from the moderately hard water was much greater than the EC20 of 9.6 mg NO_3-N/L for biomass obtained from our study. Aside from the fact that Baker et al. (2017) tested their midges using a beach-collected sand supplemented with peat as substrate whereas we tested ours over the silica sand, it is difficult to account for the vast difference in effect levels. However, Baker et al. (2017) used third instar midges (~10 d after hatching), whereas we used second instar (~7 d) midges. Previous experiments have suggested that the acute sensitivity of midge larvae was greater for earlier instar larval midges than the later instars, in some cases by several fold (Nebeker et al. 1984; Williams et al. 1986). In addition, Baker et al. (2017) fed 6 mg of TetraMin each day in static renewal (3 times/wk), whereas we fed 4 mg TetraMin twice a day on days 0 to 3 and 6 mg once daily in a flow-through diluter (every 4 h of water addition), and their control mean individual dry weight at the end of exposures was 0.156 whereas the mean control dry weight from our study was 0.174 mg. The earlier starting age and better control growth in our study may have resulted in the lower effect concentration.

For the gray treefrog, Vaala et al. (2004) reported no effect on lethal and sublethal (growth, activity) endpoints at NO_3 treatments up to 20 mg NO_3-N/L in 15-d exposures. Similarly, our longer (52-d) exposures did not result in significant reductions in survival, individual weight, and biomass at NO_3 concentrations up to 111 mg NO_3-N/L (Supplemental Data, Table S8). However, the longer test duration allowed us to detect reductions in the percentage of gray treefrogs metamorphosing at the 2 highest NO_3 concentrations (111 and 52 mg NO_3-N/L).

The toxic mechanism of NO_3 is thought to be similar to that of nitrite (NO_2) after in vivo conversion of NO_3 to nitrite (Cheng and Chen 2002). The NO_2 converts the blood pigments hemoglobin and hemocyanin to methemoglobin and methemocyanin, respectively, thereby causing a loss of oxygen-carrying capacity by these pigments (Camargo and Alonso 2006). All of the organisms tested in the present study have blood containing one of these pigments, but the midge in particular is rare among insects in possessing hemoglobin. This might account for the observed sensitivity of this particular species in the present study.

Acute SO_4 toxicity to the mussels and amphipod

Control survival in all acute exposures was 100% (Table 2) and met the acute test acceptability criterion of ≥90% control survival (ASTM International 2019a, 2019b). The EC50s for the 2 species of mussels were similar (2071 mg SO_4/L for fatmucket and 2064 mg SO_4/L for rainbow mussels) and slightly lower than the EC50 of 2689 mg SO_4/L for the amphipod (Table 2). The EC50 for fatmucket was similar to the EC50 of 2325 mg SO_4/L for the same species reported in a previous study (Wang et al. 2017). In addition, the EC50s for the fatmucket and rainbow mussel tested in the present study were within the ranges of 1338 to 2709 mg SO_4/L for 5 other species of mussels from different tribes and families tested under similar conditions (Wang et al. 2016, 2017). Another more distantly related bivalve, the fingernail clam *Sphaerium simile*, also had an SO_4 EC50 within this same range (2078 mg SO_4/L; Soucek and Kennedy 2005), whereas 2 snails (*Lymnaea stagnalis* and *Physa gyrina*) tested under similar conditions had substantially higher EC50s of 6571 and 3295 mg SO4/L, respectively (Ivey et al. 2017).

For the amphipod (*H. azteca*), several previous studies have reported SO_4 EC50s in water with a similar hardness to that used in the present study and with Cl of at least 10 mg/L, and all of the values are within a factor of 2 to the one reported here (Soucek and Kennedy 2005; Davies and Hall 2007; Soucek 2007; Elphick et al. 2011).

Chronic SO_4 toxicity to the mussel, amphipod, and fish

Mean control survival of all test species in chronic exposures ranged from 93 to 100% (Table 2) and met the chronic test acceptability criterion of ≥80% control survival of the mussel and amphipod (ASTM International 2019b, 2019c) and ≥70% control survival of fish (ASTM International 2019d). Significant reductions in lethal and sublethal endpoints were observed in tests with the 2 species of mussels (LOEC as low as 392 and 713 mg SO_4/L), the amphipod (1990 mg SO_4/L), and the fathead minnow (224 mg SO_4/L) whereas no significant effects

TABLE 2: Acute and chronic effect concentrations (95% confidence limits in parentheses) for sulfate (mg SO_4/L) in sodium sulfate exposures with fatmucket (*Lampsilis siliquoidea*), rainbow mussel (*Villosa iris*), amphipod (*Hyalella azteca*), fathead minnow (*Pimephales promelas*), and rainbow trout (*Oncorhynchus mykiss*)

Species	Test (test duration)	Age of organism at test start	Control survival (%)	Acute EC50 (mg SO_4/L)	Chronic LC20 or EC20 (mg SO_4/L)			
					Survival	Dry weight	Biomass	Reproduction
Fatmucket								
	Acute (4 d)	~10-d juveniles	100	2071 (1928–2224)	—[a]	—	—	—
	Chronic (28 d)	~35-d juveniles	100	—	1738 (1629–1789)	438 (190–1012)	662 (417–1050)	—
Rainbow mussel								
	Acute (4 d)	~15-d juveniles	100	2064 (1925–2215)	—	—	—	—
	Chronic (28 d)	~40-d juveniles	95	—	1701 (1512–1811)	384 (186–794)	395 (212–735)	—
Amphipod								
	Acute (4 d)	7 to 9-d-old juveniles	100	2689 (2316–3122)	—	—	—	—
	Chronic (42 d)	7 to 9-d-old juveniles	96	—	>1990[b]	1111 (886–1394)	1151 (913–1452)	1281 (742–1372)
Fathead minnow								
	Chronic (34 d)	<1-d embryos	93	—	1140 (951–1311)	NE[c]	377 (226–627)	—
Rainbow trout								
	Chronic (55 d)	Eyed embryos	97	—	>3240	>3240	>3240	—

[a]Not applicable.
[b]A greater-than value was reported when no exposure concentration caused an effect of >40% in acute exposures and >15% in chronic exposures relative to the control.
[c]An EC20 for weight was not estimated because of the uncertainty of the density-dependent growth effect (see Supplemental Data, Table S11).
EC20 or EC50 = 20 or 50% effect concentration; LC20 = 20% lethal concentration; NE = not estimated.

were observed in the trout test at up to 3240 mg SO_4/L (Supplemental Data, Tables S9–S12). The EC20s based on growth or reproduction were 2- to 4-fold lower than the EC20s for survival across the 4 species (Table 2). Based on the most sensitive endpoint of dry weight, the EC20s of 438 mg SO_4/L for fatmucket and 384 mg SO_4/L for rainbow mussels were >2-fold lower than the EC20 of 1111 mg SO_4/L for the amphipod and slightly greater than the EC20 of 374 mg SO_4/L for the minnows (Table 2). The similar chronic sensitivity of the 2 species of mussels was consistent with that observed in acute SO_4 exposure, but in contrast to findings in acute SO_4 exposures, the mussels were more sensitive than the amphipod in chronic SO_4 exposures.

The 28-d EC20s for fatmucket and rainbow mussels from the present study were approximately 30% lower than a 28-d EC20 of 639 mg SO_4/L based on dry weight for another unionid mussel (*L. abrupta*) from a previous study in a similar background water (Wang et al. 2016). For the amphipod, the 42-d EC20 from the present study was similar to that of the 14-d EC25 of 1056 mg SO_4/L reported by Elphick et al. (2011).

Elevated mortality of the fathead minnow was observed in the present study at the high exposures of 1600 and 3200 mg SO_4/L treatments soon after hatching on test days 4 and 5 (Supplemental Data, Table S13). This was consistent with the finding in the previous Na_2SO_4 test with the fathead minnow in a similar test water (i.e., the diluted CERC well water with the addition of K to 3 mg K/L; Wang et al. 2016). Specifically, the 7-d LC20 of 1096 mg SO_4/L (Supplemental Data, Table S13) was similar to the 7-d LC20 of 1180 mg SO_4/L in the previous study with the similar test water (Wang et al. 2016). However, the LC20s from the diluted well water at the hardness of 3 mg/L were more than 2-fold greater than the 7-d LC20 in the CERC diluted well water without an addition of K (i.e., at a lower K concentration of 1.0 mg/L; Wang et al. 2016). The results of the present study again confirmed the influence of K on Na_2SO_4 toxicity to fathead minnow embryos.

Another study was conducted recently to better understand the range of the K influence of the Na_2SO_4 on fathead minnow in an early life stage test. The test started with <24-h-old minnow embryos in unamended Lake Superior water containing 0.6 mg K/L (D. Mount, personal communication). The results of this study confirmed the high sensitivity of the minnow embryos to Na_2SO_4 at the low K. However, their additional 7-d embryo and larval survival tests with Na_2SO_4, $MgSO_4$, NaCl, or $MgCl_2$ indicated that the high sensitivity of the minnow embryos was likely associated with Na, rather than SO_4 (D. Mount, unpublished data). More studies may be needed to evaluate the effect of Na on the minnow embryo and the growth of larval and juvenile fish at a higher concentration of K (e.g., 3 mg K/L).

As observed in the previous Na_2SO_4 toxicity tests at the low K of 1.0 mg/L (Wang et al. 2016), the elevated mortality of the fathead minnow in the present study at the 3.0 mg K/L level was primarily observed soon after hatch, and survival after the first 7-d exposure was stable over the rest of the 34-d exposure. Among the 4 treatments with no effect on survival, the 34-d growth (length and dry wt) in the 2 high treatments was significantly reduced relative to the control (Supplemental Data, Table S11). The LOEC of 371 mg SO_4/L based on the growth

endpoints from the present study in the higher K water was greater than the LOECs based on growth from the previous study in the low K water (74–215 mg SO_4/L; Wang et al. 2016). The lower sensitivity based on the growth endpoint in the higher K water was consistent with the lower sensitivity based on the survival in the higher K water.

Density-dependent growth was observed in a previous study (Wang et al. 2016) and the present study (Supplemental Data, Table S11); mean length and dry weight of surviving minnows (≤53%) in the high treatment were greater than those in the control, likely due to less intraspecific competition and more available food. Although the use of biomass (a combined effect of survival and growth) would limit the influence of the density-dependent growth, additional studies are needed to further evaluate the effects of Na_2SO_4 on fish growth (e.g., at different feeding rates), to determine whether the growth is a robust endpoint to establish chronic toxicity thresholds for fathead minnow.

In contrast to the high sensitivity of the fathead minnow, the embryonic and hatched trout survived and grew well in all treatments of up to 3240 mg SO_4/L over 55 d of exposure (Table 2; Supplemental Data, Table S12). However, in a previous 21-d Na_2SO_4 toxicity test starting with eyed embryos of rainbow trout in test water with a hardness of 100 mg/L as $CaCO_3$ (i.e., the same fish starting stage and water hardness as the present study), elevated mortality was observed in high treatments of up to 2000 mg SO_4/L, with an LC20 of 597 mg SO_4/L (Appendix B in British Columbia Ministry of Environment 2013). The K in the test water was not reported for the specific test, but a concentration of approximately 2 mg/L was reported for the test water in an earlier study (Appendix A in British Columbia Ministry of Environment 2013). It is uncertain whether the lower K in the test water resulted in the lower effect concentration in the previous study.

Acute-to-chronic ratios

Acute-to-chronic ratios (ACRs) for the species tested in the present study were calculated based on the acute LC50/EC50 and chronic EC20 for the most sensitive endpoint. The ACRs for NO_3 ranged from 13 to 39 for the midge, mussel, and treefrog, and the ACRs for SO_4 ranged from 2.4 to 5.4 for the amphipod and 2 mussel species (Table 3). The ACRs of 4.7 and 5.4 for the 2 mussels were similar to the ACR of 3.2 for another mussel species (*L. abrupta*) tested in the previous study (Wang et al. 2016).

Species sensitivity

To compare the species sensitivity among freshwater organisms, species mean acute values (SMAVs) were calculated as a geometric mean of LC/EC50s for survival of each species and species mean chronic values (SMCVs) were calculated as a geometric mean of chronic LC/EC20s (or IC25s and MATCs when LC/EC20s were not available; see details in the *Data analyses* section) for the most sensitive endpoint of each species. The SMAVs and SMCVs were ranked and plotted in cumulative distributions (Figures 1 and 2). All available data in the compiled NO_3 databases were used for the sensitivity comparison because of the uncertainty of any hardness influence on the toxicity of $NaNO_3$, as discussed previously in the *Acute NO_3 toxicity to the mussel, midge, fish, and amphibian* section. The NO_3 data had a hardness range from 44 to 298 mg/L as $CaCO_3$. However, because the toxicity of Na_2SO_4 to aquatic organisms can be influenced by water hardness (Mount et al. 1997, 2016; Soucek and Kennedy 2005; Davies and Hall 2007; Soucek 2007; Elphick et al. 2011; Soucek et al. 2018), only the data in the compiled SO_4 database within a hardness range from 80 to 110 mg/L as $CaCO_3$ were used for the species sensitivity comparisons, to minimize the hardness influence. In addition, because the SO_4 EC20s for the fish (fathead minnow and rainbow trout) from the present study in the higher K water were more than 2-fold greater than the EC20s for the same fish species from the previous study in the lower K water (British Columbia Ministry of Environment 2013; Wang et al. 2016), only EC20s from the tests in the lower K water were used for the calculation of the SMCV, indicating the worst-case scenario for the fish species.

TABLE 3: Acute EC50 and chronic EC20 for sodium nitrate and sodium sulfate, and acute-to-chronic ratio in exposures with midge (*Chironomus dilutus*), fatmucket (*Lampsilis siliquoidea*), rainbow mussel (*Villosa iris*), amhipod (*Hyalella azteca*), rainbow trout (*Oncorhynchus mykiss*), or gray treefrog (*Hyla versicolor*)

	Nitrate (mg NO_3-N/L)			Sulfate (mg SO_4/L)		
Species	EC50	EC20	ACR	EC50	EC20	ACR
Midge	189	9.6	20	—[a]	—	—
Fatmucket	665	17	39	2071	438	4.7
Rainbow mussel	—	—	—	2064	384	5.4
Amphipod	—	—	—	2689	1111	2.4
Rainbow trout	>883	>38	NC	—	—	—
Gray treefrog	601	47	13	—	—	—

[a]Not applicable.
EC20 or 50 = 20 or 50% effect concentration; ACR = acute-to-chronic ratio; NC = not calculated.

In the acute NO_3 species sensitivity distribution (Figure 1A), 4 insects (1 mayfly, 2 caddisflies, and 1 midge) ranked as the 4 most sensitive species. A few invertebrates (such as mollusks, amphipods, and cladocerans) also ranked relatively low (<50th percentile) and were more sensitive than fish and amphibians. Similarly, invertebrates were more sensitive than fish and amphibians in the chronic NO_3 species sensitivity distribution (Figure 1B). However, the midge became the most sensitive species in chronic exposures, followed by a mussel, amphipod, and mayfly.

In the acute SO_4 species sensitivity distribution (Figure 2A), 1 mayfly, 2 mussels, and 1 amphipod were the 4 most sensitive species, whereas the midge became the least sensitive species. Fathead minnow was the only vertebrate in the database and was not sensitive relative to most invertebrates. However, fathead minnow became the most sensitive species in chronic SO_4 database, followed by 1 mayfly and 2 mussels. Rainbow trout was also relatively sensitive (ranked as the fifth most sensitive species at the 42th percentile). Again, the sensitivity of the fish early life stages to Na_2SO_4 was likely influenced by K in test water. If the chronic values from the present study with the higher K test water were used to calculate the SMCVs, the fathead minnow would rank as the second most sensitive species at the 16th percentile, and the

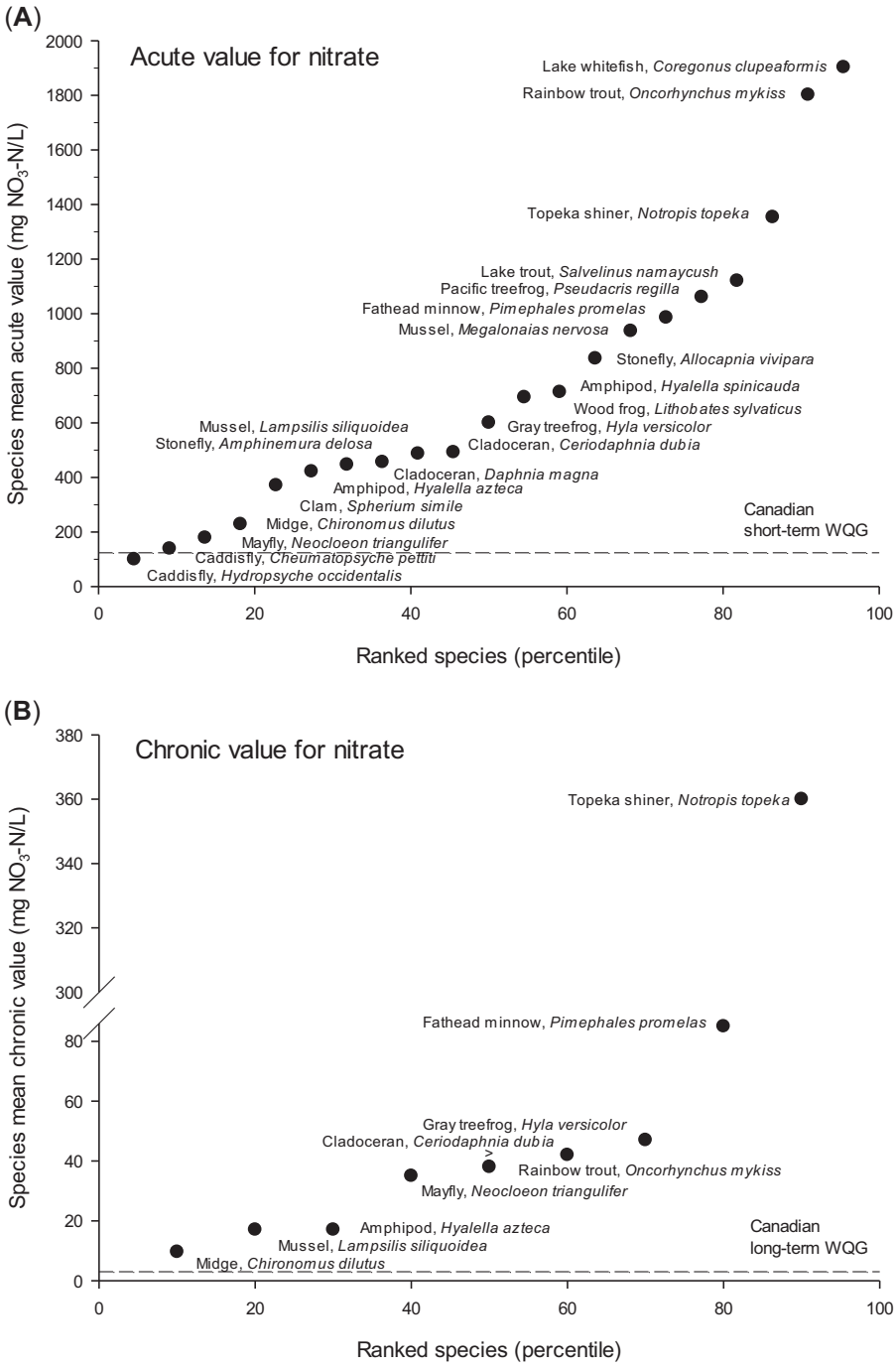

FIGURE 1: Ranked freshwater species mean acute values (**A**) and species mean chronic values (**B**) in a compiled database for nitrate (tested as $NaNO_3$). The symbol > above the chronic value for rainbow trout indicates a larger-than value (i.e., the actual rank of the trout would be higher). Dashed line indicates the Canadian water quality guidelines (WQG) for nitrate ion (Canadian Council of Ministers of the Environment 2012).

rainbow trout would rank as the 10th most sensitive species at the 83rd percentile.

Implications for environmental guideline values

Canadian water quality guidelines for NO_3 were published in 2012, with 124 mg NO_3-N/L for short-term exposure and 3.0 mg NO_3-N/L for long-term exposure (Canadian Council of Ministers of the Environment 2012). All the SMAVs in Figure 1A and SMCVs in Figure 1B in our compiled NO_3 databases were above the Canadian water quality guideline except for 2 caddisflies in acute exposure (Figure 1A). The British Columbia water quality guideline for SO_4 was developed based on subchronic and chronic toxicity data for 4 categories of water hardness (British Columbia Ministry of Environment 2013). The British Columbia water quality guideline for moderately soft/hard to hard water (76–180 mg/L) was 309 mg SO_4/L. All the SMAVs and the most SMCVs in our compiled SO_4 databases at hardness

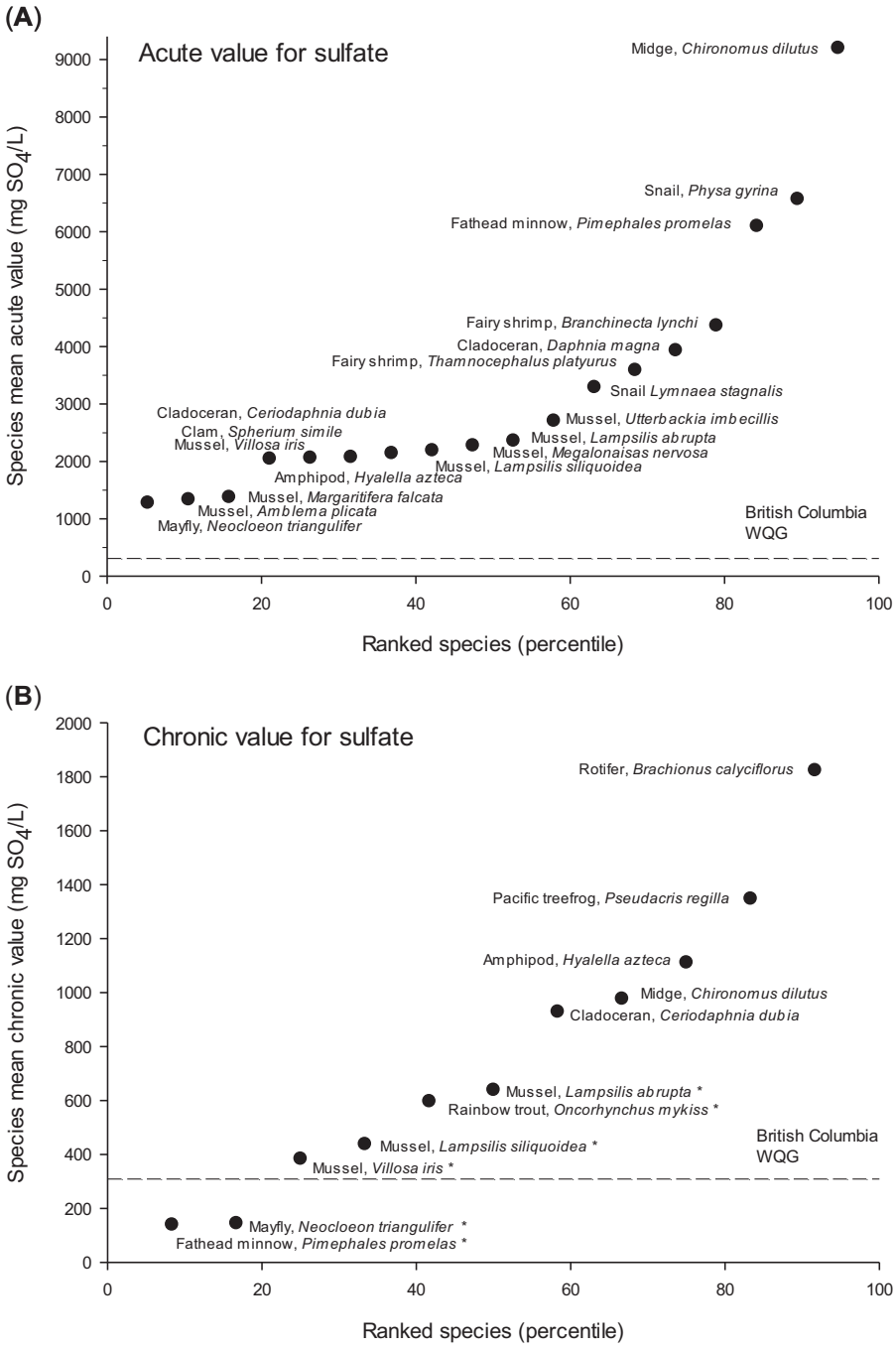

FIGURE 2: Ranked freshwater species mean acute values (**A**) and species mean chronic values (**B**) in a compiled database for sulfate (tested as Na_2SO_4) generated from test waters at a hardness range from 80 to 120 mg/L as $CaCO_3$. Note: The 20% effect concentrations (EC20s) for fathead minnow and rainbow trout tested in a lower potassium water were used for the calculation of the species mean chronic values in this figure (see the *Species sensitivity* section). Dashed line indicates the British Columbia water quality guideline (WQG) for sulfate in moderately soft/hard to hard water (76–180 mg/L as $CaCO_3$; British Columbia Ministry of Environment 2013). An asterisk indicates a species mean chronic value at or below the Illinois State water quality standard for sulfate (Illinois Pollution Control Board 2011) after adjusting for the hardness and chloride levels in each test water. (A single line for the Illinois State water quality standard cannot be presented in this figure because these standards are dependent not only on hardness but also on chloride, and are different among various test waters; see details in the *Implications for environmental guideline values* section).

80 to 110 mg/L were above the British Columbia water quality guideline (Figure 2); however, the chronic values for fathead minnow and mayfly (*N. triangulifer*) were far below the water quality guideline (Figure 2B). The Illinois State water quality standards for SO_4 are dependent on hardness and Cl, and provide "must be met at all times" values based on acute toxicity data (Illinois Pollution Control Board 2011). All the acute values in our compiled acute toxicity dataset (Figure 2A) were above the Illinois water quality standards, whereas the chronic values for 6 species in the compiled chronic database in

Figure 2B were at or below the Illinois water quality standards "must be met at all times" value adjusted for water quality characteristics. Therefore, including the toxicity data from the present study would likely lower the Canadian water quality guideline for NO_3 and the British Columbia water quality guideline and Illinois water quality standards for SO_4.

Overall, the results of the present study indicate that both acute and chronic toxicity data should be used to refine or develop environmental guidance values for NO_3 and SO_4. Including the species tested in the present study into the existing databases for NO_3 and SO_4 would meet the USEPA minimum data requirement of at least 8 taxonomic groups (Stephan et al. 1985) for developing national WQC and state water quality standards. In addition, the results from the present study confirmed the influence of K on chronic Na_2SO_4 toxicity to the fathead minnow reported in the previous study (Wang et al. 2016). Therefore, environmental guidance values for SO_4 might need to address the influence of K on SO_4 toxicity, particularly in waters with lower concentrations of K. However, a more recent study has indicated that the high sensitivity of the minnow embryos was likely associated with Na, rather than SO_4 and, importantly, it is rare that low K (<3 mg/L) is present in surface waters with elevated Na (D. Mount, unpublished data). These findings also need to be considered for developing the guideline values.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at https://doi.org/10.1002/etc.4701.

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